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Small GTPase Rho5 is a functional homologue of Rho1, which controls cell shape and septation in fission yeast

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Abstract The small GTPase Rho1 plays an essential role in controlling the organization of the actin cytoskeleton and synthesis of the cell wall in the fission yeast *Schizosaccharomyces pombe*. Here we studied the role of Rho5 whose primary structure is very similar to that of Rho1. It was found that elevated expression of Rho5 was able to compensate for the lethality of cells lacking Rho1. Rho5 was localized to the ends of interphase cells and the mid-region of mitotic cells. Overexpression of Rho5 caused depolarization of F-actin patches and abnormal formation of the cell wall, as did Rho1. Although *rho5*$^+$ was not essential for maintaining the cell shape, *rho1 rho5*-double null cells showed more severe defects in cell viability than *rho1*-null cells. Thus, it is likely that Rho5 has an overlapping function with Rho1 in controlling cell growth and division in *S. pombe*.

Keywords: Actin cytoskeleton; Cell wall; Morphogenesis; Rho; Septation

1. Introduction

The fission yeast *Schizosaccharomyces pombe* is an excellent organism for studying the molecular mechanisms, which controls cell morphogenesis and cell division [1,2]. *S. pombe* cells are cylindrical and are surrounded by a rigid cell wall. The actin cytoskeleton is organized as cortical patches localized at the growing ends of the interphase cell, where the cell wall is synthesized, and also as cables that run the length of the cell [3,4]. Using a protoplast regeneration system, it has been shown that the polarized localization of F-actin patches is required for cell wall synthesis [5]. During cell division, F-actin patches disappear from the cell ends and the F-actin ring is formed in the middle cortex of the cell. Following contraction of the F-actin ring, F-actin patches are newly formed around the division site and the primary septum is synthesized. After complete formation of the primary septum, secondary septa are formed on both sides of the primary septum. Finally, daughter cells are separated by digestion of the primary septum after complete formation of the secondary septa. The cells then undergo polarized growth first at the old end and then at both the old and the new ends. F-actin patches are seen to accumulate at the end(s) where the cell grows. Thus, coordinated control between the organization of the actin cytoskeleton and synthesis of the cell wall is probably essential for cell morphogenesis and cytokinesis in fission yeast.

It has been considered that Rho-family small GTPases localize to the membrane through their isopenyl C-terminus and function as a molecular switch for controlling the organization of the actin cytoskeleton, membrane traffic, cell cycle progression, and stress response [6,7]. In order to clarify the function of Rho in cell morphogenesis and cytokinesis in fission yeast cells, we isolated cDNAs encoding Rho-family small GTPases [8] and characterized their functions. Rho1 is a functional homologue of human RhoA and budding yeast Rho1p and regulates the activity of β1,3-glucan synthase, the cellular expression level of PKC-related protein kinases Pck1 and Pck2, as well as the localization of F-actin patches [9–13]. Rho2 has been shown to be involved in controlling cell morphogenesis probably by regulating the synthesis of α-glucan through a Pck2 pathway [14,15]. In addition, it has been reported that Rho3 binds to a formin-family protein, For3, and controls both the organization of microtubles and the actin cytoskeleton [16]. Rho4 is required to achieve cell separation and maintain cell shape in a manner different from other Rho proteins [17,18]. On the other hand, the function of Rho5 has not yet been elucidated. Thus, we investigated the cellular role of Rho5 in fission yeast in this report.

2. Materials and methods

2.1. Strains, media and genetic techniques

The *S. pombe* strains used in this study were as follows: *leu1-32* (genotype; *h*$^-$ *leu1-32*), *JY741* (*h*$^-$ *ade6-M216 leu1-32 ura4-D18*) and *JY746* (*h*$^+$ *ade6-M210 leu1-32 ura4-D18*). The media used have been described previously [19]. Complete medium YEA and minimum medium EMM were used to grow *S. pombe* strains. MEA was used for the induction of conjugation and sporulation. All plates contained 2% agar. Standard procedures for *S. pombe* genetics were followed according to Moreno et al. [19] and Alfa et al. [20]. Standard methods were used for DNA manipulations [21].

2.2. Creation of the rho5-null allele

An *Spe*I-digested 2.8 kb fragment of genomic DNA containing *rho5*$^+$ was cloned from the *S. pombe* genomic library [12] using *rho5*$^+$ cDNA (GenBank Accession No.: D86612) as a probe. To disrupt the *rho5*$^+$ gene, we replaced an *Sac*I–*Sac*I fragment with the *ura4*$^+$ gene in the open reading frame of *rho5*$^+$ genomic clone (5–131 amino acid residues removed). A fragment produced by digestion with

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*Spe*I was then used to transform a diploid constructed by mating *JY741* and *JY746* strains. Correct integration was verified by Southern blotting.

2.3. Gene expression in fission yeast

rho5$^+$ cDNAs were amplified using oligonucleotides (R5N (5′-cccatatgactactgagctccgtcgtaaattgg-3′) and R5C (5′-ggggatcctcagagtaagatacagtg-3′)), which contained *Nde*I or *Bam*HI sites at the 5′ and 3′ ends. The PCR-amplified fragment was ligated to pREP1 [22], which had been pretreated with *Nde*I and *Bam*HI. pREP1 has the strongest promoter, while pREP81 has the weakest one. pREP102-1rho5 was constructed by replacing the *LEU2* marker gene on pREP1rho5 with *ADE2*. The expression of exogenous genes from these plasmids was repressed by adding 4 μM thiamine to the medium.

To introduce a mutation in the phosphate-binding loop of Rho5, we first made an *Sph*I site at +46 bp of its ORF: this nucleotide substitution was a silent substitution. Next, we amplified fragments of *rho5* mutants using nucleotides (*rho5G15V*; R5N and 5′-accgcatgcaacatcgcc-3′, and *rho5T20N*; 5′-ggtgcatgcggtaaaaattgcttgt-3′ and R5C) and replaced *Nde*I and *Sph*I- or *Sph*I and *Bam*HI regions of *rho5*$^+$ cDNA. The *rho5C197S* mutant was produced by PCR using oligonucleotides (R5N and 5′-ggggatcctcagagtaagatactgtgcttcttc-3′). These *rho5* mutants were inserted into the *Nde*I and *Bam*HI sites of pREP1.

2.4. Characterization of a phenotype of rho1 rho5-double null cells

To make *rho1 rho5*-double null cells, we first removed *ura4*$^+$ from *rho5*-null cells (*KNR502*; *h*$^-$ *ade6-M216 leu1-32 ura4-D18 rho5::ura4*$^+$) by transforming with the *Spe*I fragment of an *rho5*$^+$ genomic clone lacking a *Sac*I–*Sac*I region and pREP81rho1 [12]. Transformants were inoculated on EMM containing adenine, uracil and 5-fluoroorotic acid (Sigma). After correct integration was verified by Southern blotting, the *rho5*-null strain containing pREP81rho1 was twice backcrossed with the wild-type strain. Next, we made a diploid constructed by mating strains, *KNR506* (*h*$^-$ *ade6-M216 leu1-32 ura4-D18 rho5-D1*) and *KNR507* (*h*$^+$ *ade6-M210 leu1-32 ura4-D18 rho5-D1*), containing pREP81rho1, and disrupted the entire ORF of *rho1*$^+$ by transforming with a 2.8-kb *Xho*I–*Nde*I fragment containing *ura4*$^+$ in the −30 to +940 bp region of *rho1*$^+$. After correct integration was verified by Southern blotting, we isolated *rho1 rho5*-double null cells containing pREP81rho1 by dissecting spores of the diploid.

To compare the phenotype of *rho1*-null cells and *rho1 rho5*-double null cells, both strains were grown in EMM to the mid-log phase. After adding thiamine to the media, each cell aliquot was isolated and stained with phloxin B (Sigma).

2.5. Fluorescence microscopy and electron microscopy

Cells were stained with Calcofluor (Sigma) or Rhodamine-phalloidin (Molecular Probes, Inc.) as described previously [20] and viewed using a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Inc.) equipped with a Plan Apochromat ×63 lens and photographed on Kodak T-MAX ASA 400 film.

Electron microscopic observation was carried out according to Nakano et al. [12].

2.6. Expression and observation of GFP fusion proteins

Green fluorescence protein was amplified by PCR from pGFP (Clontech Lab.), and the amplified fragment was digested with *Pst*I and *Eco*RI as previously reported [12]. *rho5*$^+$ cDNA was amplified using oligonucleotides (5′-gggaattcatgactactgagctccgtcg-3′ and 5′-ggggatcctcagagtaagatacagtg-3′), and the amplified fragment was digested with *Eco*RI and *Bam*HI. These fragments were ligated together to *Pst*I- and *Bam*HI-treated pART1 [23]. *rho5*-null cells were transformed using this plasmid. The transformant was grown in EMM at 30 °C to mid-log phase and examined by fluorescence microscopy.

3. Results

3.1. Rho5 possesses a function similar to Rho1

Rho5 is very similar to *S. pombe* Rho1 in its primary structure (Fig. 1A). It has been demonstrated that Rho1 is essential for cell viability and controls formation of the cell wall and

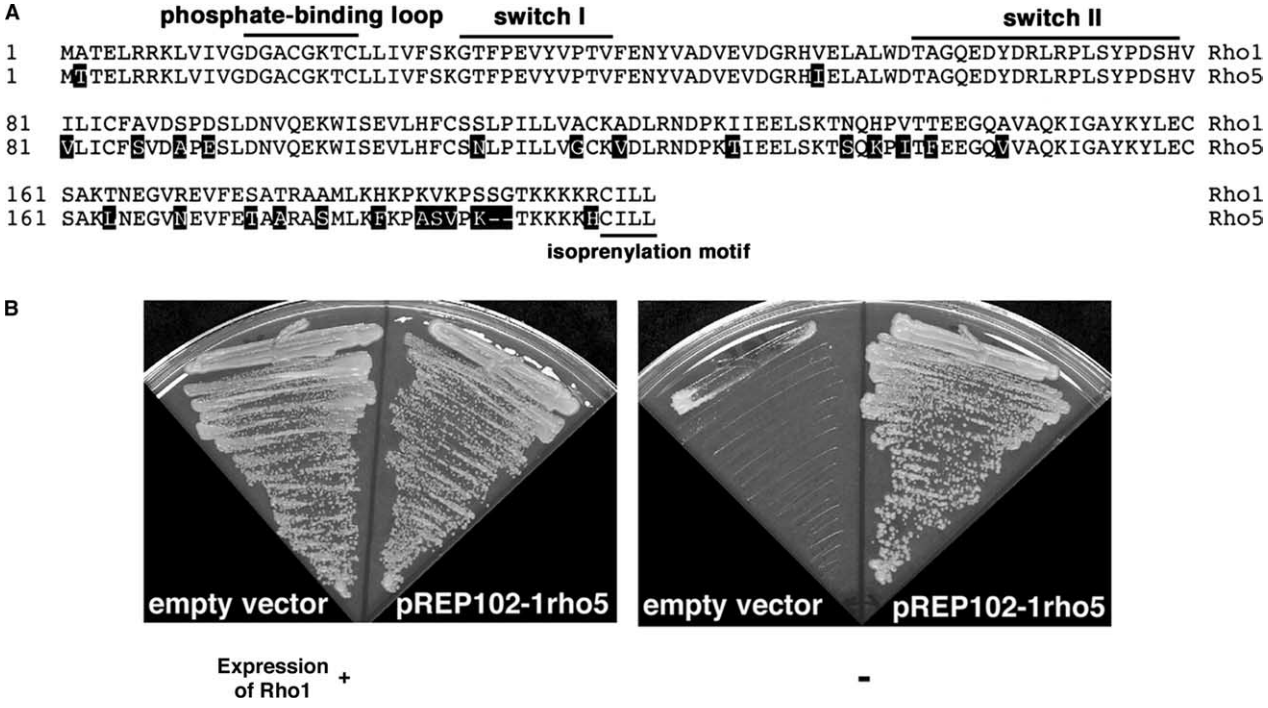

Fig. 1. *rho5*$^+$ encodes a protein similar to Rho1 in fission yeast. (A) Comparison of the putative amino acid sequences of Rho1 and Rho5. Amino acids not conserved between them are reversed in the Rho5 sequence. (B) Effect of Rho5 expression in the absence of Rho1. *rho1*-null cells containing pREP81rho1 were transformed with pREP102 or pREP102-1rho5. Each of the transformants was streaked on EMM plates with or without thiamine at 30 °C for 3 days.

organization of the actin cytoskeleton [10,12]. We then examined whether the growth defect in *rho1*-null cells was rescued by increasing expression level of Rho5. *rho1*-null cells expressing Rho1 from pREP81 which contains the *nmt1* promoter weakened by a mutation, were able to grow normally in the absence of thiamine but not in its presence (data not shown). This strain was transformed with pREP102-1rho5 which had the *rho5*$^+$ cDNA downstream of the normal *nmt1* promoter. The transformant grew normally in the presence of thiamine where expression of Rho1 was repressed while that of Rho5 was not completely repressed because the normal *nmt1* promoter was much stronger than the mutated one. The cells containing the empty vector did not grow under the same condition (Fig. 1B). This indicated that Rho5 compensated for a defect caused by Rho1 depletion.

It has been observed that Rho1 overexpression produces abnormal cell shapes by inducing depolarization of F-actin patches and exaggerated formation of cell wall [9,12]. To examine whether the Rho5 function overlaps with Rho1, we investigated phenotype of cells overexpressing Rho5. It was found that Rho5 overexpression caused a phenotype similar to that of Rho1. A significant population of the cells overexpressing Rho5 had a dumpy shape (89%) in which localization of F-actin patches were partially disordered (Fig. 2A). Moreover, population of cells containing septum was increased after overexpression of Rho5 (27% of total cells) as compared to that of the control cells (18%). Multi-septated cells were sometimes seen in the cells overexpressing Rho5 (1%), while multiple septa was never seen in the control cells (Fig. 2A, arrow). In addition, the cell wall in the cells overexpressing Rho5 (0.15 ± 0.04 μm, $n = 23$ cells) was thicker than that of the control cells (0.12 ± 0.03 μm, $n = 10$) (Fig. 3). The cell wall of *S. pombe* cells is composed of two layers; the inner layer with low electron density mainly contains β1,3-glucan while the outer layer with high electron density contains α1,3-glucan (see inset of a control cell). In contrast, those layers were obscure in the cells overexpressing Rho5 (Fig. 3, arrows). Thus, Rho5 is possibly involved in organization of the actin cytoskeleton and cell wall synthesis like Rho1.

Next, we investigated effect of expression of Rho5 mutant proteins in wild-type cells. We produced five site-specific mutations in the phosphate-binding loop and/or the isoprenylation motif of Rho5 [24]. Rho5G15V was considered to be a constitutively active mutation (GTP-bound form), while Rho5T20N a dominant-negative mutation (nucleotide-free form). Rho5C197S was expected not to be isoprenylated by isoprenyl transferase. The expression of either Rho5G15V or Rho5T20N strongly inhibited cell growth as compared to that of the wild-type protein, while that of Rho5C197S did not affect the growth. On the other hand, the C197S mutation in addition to the G15V or T20N mutation canceled the inhibitory effect the latter (Table 1). Thus, isoprenylation at

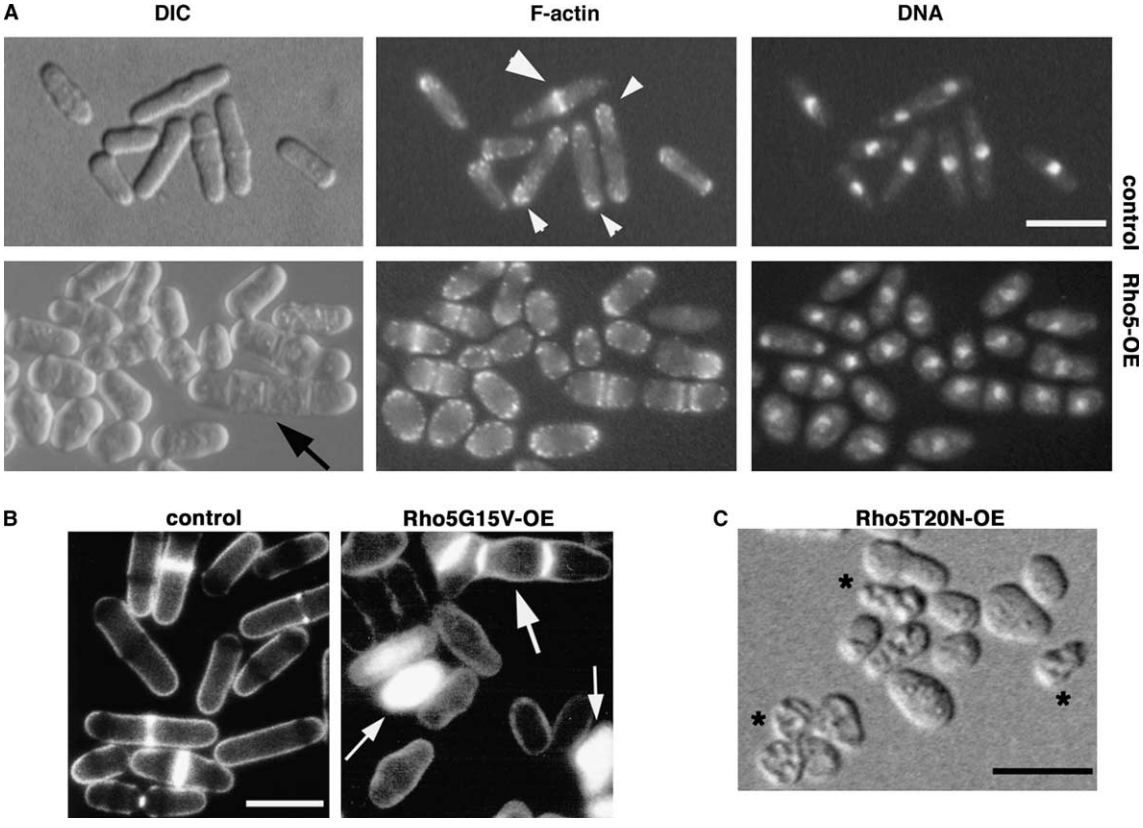

Fig. 2. Phenotype of cells overexpressing Rho5 or its mutant proteins. (A) Wild-type cells carrying pREP1 or pREP1rho5 were grown at 30 °C for 20 h in EMM without thiamine. Images of cells stained with Rhodamine-phalloidin (middle) and DAPI (right) are shown. An arrow in the DIC image (left) indicates a multi-septated cell. The large arrowhead and small arrowheads indicate the F-actin ring and patches, respectively. (B) Morphology of cells overexpressing Rho5G15V. Wild-type cells carrying pREP1 or pREP1rho5G15V were grown at 30 °C for 20 h in EMM without thiamine and stained with Calcofluor. Small arrows indicate heavy Calcofluor staining throughout the cell cortex. A large arrow indicates a multi-septated cell. (C) Morphology of cells overexpressing Rho5T20N. Wild-type cells carrying pREP1rho5T20N were grown at 30 °C for 20 h in EMM without thiamine. Asterisks indicate shrunken cells. Bars represent 10 μm.

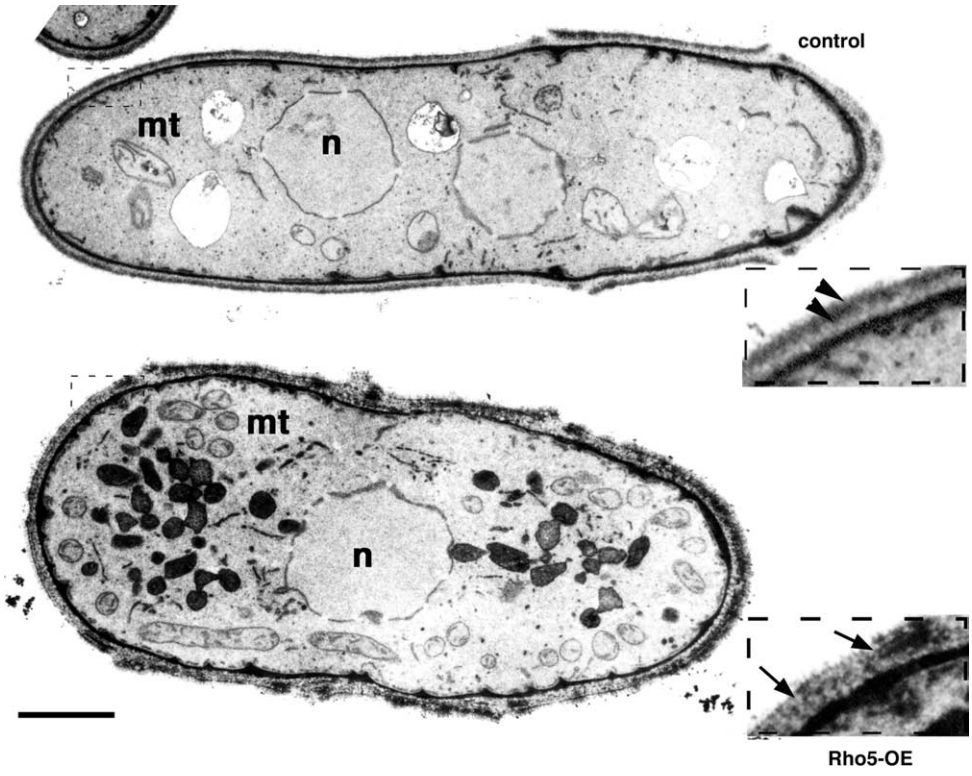

Fig. 3. Electron micrographs of a wild-type cell and a cell overexpressing Rho5. The wild-type cell carrying pREP1 (top) or pREP1rho5 (bottom) was grown at 30 °C for 20 h in EMM without thiamine. Insets are magnified images of regions surrounded by dot lines. Arrowheads indicate two layers in the cell wall of a control cells. Arrows indicate abnormal structure of cell wall induced by overexpression of Rho5. n, nucleus. mt, mitochondrion. Bar represents 1 μm.

the C-terminus of Rho5 is probably required for its localization on the membrane as in the case of other Rho proteins. The expression of Rho5G15V from pREP1 induced appearance of cells strongly stained with Calcofluor (41% of total cells) (Fig. 2B), suggesting that active Rho5 induced cell-wall synthesis. The cells overexpressing Rho5G15V seemed to be a live since the cells were rarely stained with phloxin B, a marker dye for a dead cell (data not shown). On the other hand, the expression of Rho5T20N from pREP1 induced appearance of shrunken cells (Fig. 2C). It was found that most of these cells were stained with phloxin B (data not shown), indicating that overexpression of Rho5T20N induced cell death. This phenotype was very similar to that of *rho1*-null cells or cells overexpressing the dominant-negative *rho1* mutant, Rho1T20N [10,12]. These results strongly suggested that the function of Rho5 in cell morphogenesis overlaps with that of Rho1.

3.2. Rho5 functions in cell growth together with Rho1

We isolated a *rho5*$^+$ genomic DNA by plaque hybridization using a *rho5*$^+$ cDNA probe (see Section 2). Sequence comparison with the cDNA showed that the ORF of *rho5*$^+$ contained a single intron. To investigate the effect of *rho5*$^+$ disruption in fission yeast cells, we created a null allele by replacing 63% of the N-terminal ORF with a *ura4*$^+$ selectable marker and then used this fragment to replace one copy of *rho5*$^+$ in a diploid strain. Tetrad analysis showed that *rho5*$^+$ was not essential for cell viability. The morphology of *rho5*-null cells was normal at 25–37 °C like wild-type cells (Fig. 4A). Moreover, the actin cytoskeleton was normally organized in the *rho5*-null cells (Fig. 4A). Therefore, the function of Rho5 is dispensable for growth and morphogenesis of *S. pombe* cells, possibly because Rho1 can substitute for Rho5 when it does not work. Next, we estimated expression level of Rho1 in *rho5*-null cells since it was possible that the Rho1 expression was elevated in the *rho5*-null cells. However, there was no difference in the expression levels of Rho1 between wild-type cells and *rho5*-null cells (data not shown).

We next investigated a phenotype of *rho1 rho5*-double null cells expressing Rho1 from pREP81rho1. *rho1*-null cells containing pREP81rho1 underwent several rounds of cell division

Table 1
Effects of expression of Rho5 and its mutants on the growth of *S. pombe* cells

Thiamine	Control	Rho1	Rho5					
			WT	C197S	G15V	G15V/C197S	T20N	T20N/C197S
4 μM	++	++	++	++	++	++	++	++
None	++	–	+	++	–	++	–	++

Wild-type cells containing pREP1 inserted into each cDNA were incubated on EMM plates with or without thiamine at 30 °C for 3 days. The colony size is represented by ++, +, and – for normal, small, and tiny, respectively. An empty vector was used as a control.

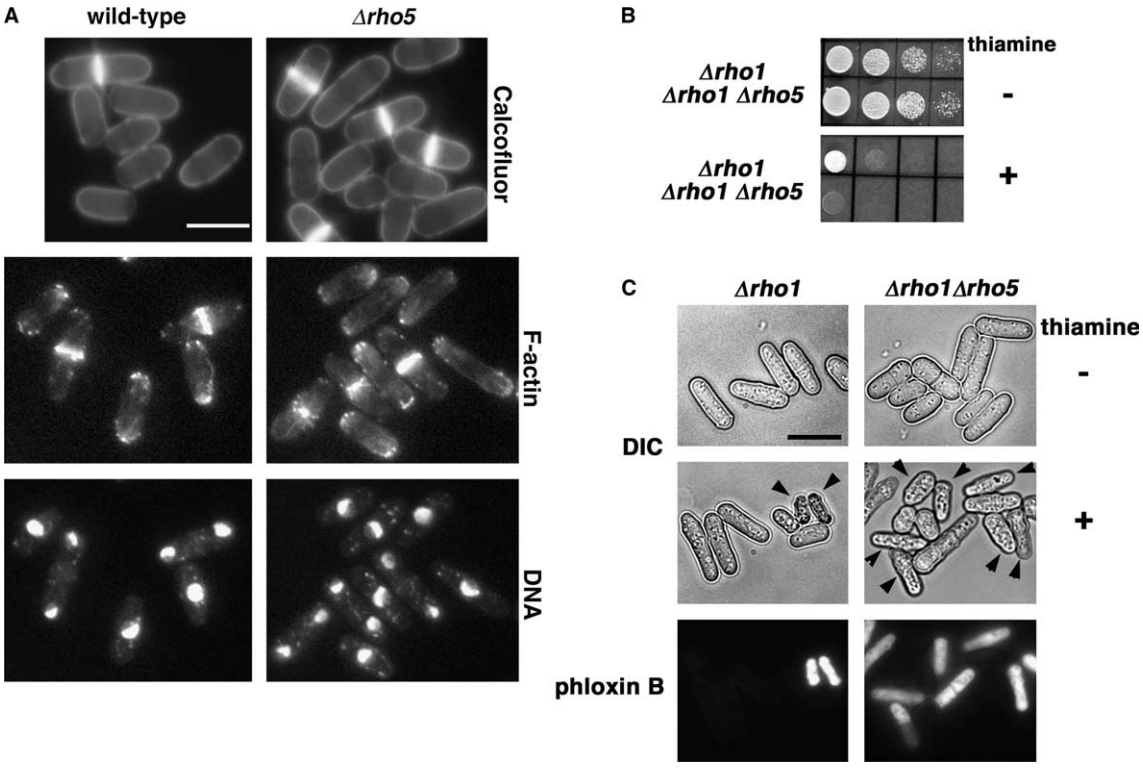

Fig. 4. Rho5 functions redundantly with Rho1 in cell growth. (A) Cell shape and organization of F-actin in *rho5*-null strain. Wild-type and *rho5*-null cells were grown to the mid-log phase at 25 °C in YE and stained with Calcofluor (top) or Rhodamin-phalloidin (middle) and DAPI (bottom). (B) Viability of cells lacking Rho1 and/or Rho5. *rho1*- and *rho1 rho5*-double null cells carrying pREP81rho1 were grown to 8×10^6 cells/ml at 25 °C in EMM without thiamine. After each culture was diluted 1-, 10-, 100- or 1000-fold with EMM, 5 μl of the cultures were spotted on EMM plates with or without thiamine. Photographs were taken after incubating the plates at 25 °C for 4 days. (C) *rho1*- and *rho1 rho5*-double null cells carrying pREP81rho1 were incubated at 25 °C for 10 h in EMM with or without thiamine. The cells incubated with thiamine were stained with phloxin B for detection of dead cells. Arrowheads indicate shrunken cells. Bar represents 10 μm.

before they ceased to grow in the presence of thiamine, because it took several hours until expression level of Rho1 was decreased enough to exhibit a terminal phenotype. Therefore, a cluster of cells was formed even in the presence of thiamine where a large number of *rho1*-null cells containing pREP81rho1 were spotted (Fig. 4B). In contrast, the *rho1 rho5*-double null cells containing pREP81rho1 showed a more severe growth defect in the presence of thiamine. It was also observed that a population of dead cells was significantly increased when the Rho1 expression was suppressed in the *rho1 rho5*-double null cells compared with the *rho1*-null cells (Fig. 4C and Table 2). Therefore, Rho5 seemed to contribute to maintaining cell viability for a short period when the activity of Rho1 is decreased.

3.3. Localization of Rho5

We investigated localization of green fluorescent protein (GFP)-fused Rho5 in *rho5*-null cells (Fig. 5). In interphase cells, GFP-Rho5 was seen to be concentrated at the periphery of the cell ends. On the other hand, in mitotic cells, the GFP signal was accumulated at the mid-region of the cell and then invaginated as septation proceeded. These localizations of

Table 2
Population of dead cells (%) after the addition of thiamine as revealed by phloxin B staining

Strains	0 h	8	10	14	18
Δ*rho1*	6.4	14.5	18.2	29.3	65.8
Δ*rho1* Δ*rho5*	5.4	40.9	54.3	60.1	76.2

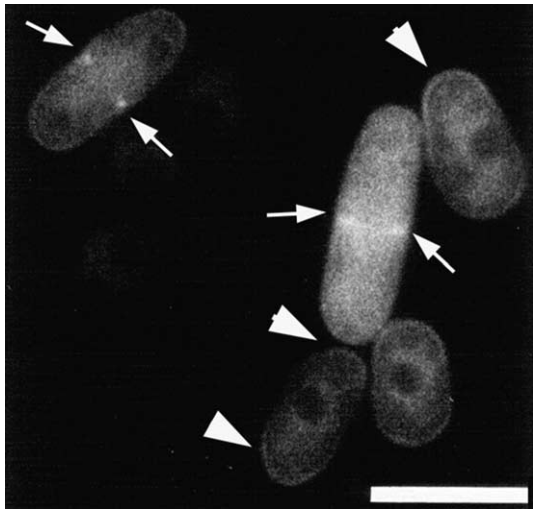

Fig. 5. Localization of GFP-Rho5. *rho5*-null cells containing pART1GFP-Rho5 were grown at 25 °C in EMM to the mid log phase and examined under a fluorescence microscope. Arrowheads and arrows indicate the localization of Rho5 at the cell ends and the middle of the cells, respectively. Bar represents 10 μm.

GFP-Rho5 were similar to those of Rho1 reported previously [12].

4. Discussion

Rho5 is likely involved in controlling cell-wall formation and organization of the actin cytoskeleton in *S. pombe* as well as Rho1. Its activity seemed to be regulated by bound guanine nucleotides and its localization may be regulated by the C-terminal isoprenylation. Rho5 serves as a functional homologue of Rho1, since the growth defect of *rho1*-null cells was suppressed by increasing the expression level of Rho5 and *rho1 rho5*-double null cells showed a severer growth defect than that of *rho1*-null cells. Rho5 was localized to the cell ends during interphase and the division site of mitotic cells like Rho1. However, *rho5*$^+$ was different from *rho1*$^+$ in that it was not required for cell growth and the maintenance of cell shape while *rho1*$^+$ is essential. Why are these genes different in the requirement for the cell? It is possible that the expression level of *rho1*$^+$ is much higher than that of *rho5*$^+$. This idea is supported by the fact that we have previously been able to isolate only 2 clones of *rho5*$^+$ cDNA against 34 clones of *rho1*$^+$ cDNA in the same screening process [8,17]. It is therefore likely that Rho1 is more important for the growth of the cell than Rho5.

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